

Three-dimensional positioning of genes in mouse cell nuclei

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Abstract To understand the regulation of the genome, it is necessary to understand its three-dimensional organization in the nucleus. We investigated the positioning of eight gene loci on four different chromosomes, including the β -globin gene, in mouse embryonic stem cells and in in vitro differentiated macrophages by fluorescence in situ hybridization on structurally preserved nuclei, confocal microscopy, and 3D quantitative image analysis. We found that gene loci on the same chromosome can significantly differ from each other and from their chromosome territory in their average radial nuclear position. Radial distribution of a given gene locus can change significantly between cell types, excluding the possibility that positioning is determined solely by the DNA sequence. For the set of investigated gene loci, we found no relationship between radial distribution and local gene density, as it was described for human cell nuclei. We did find, however, correlation with other genomic properties such as GC content and certain repetitive elements such as long

terminal repeats or long interspersed nuclear elements. Our results suggest that gene density itself is not a driving force in nuclear positioning. Instead, we propose that other genomic properties play a role in determining nuclear chromatin distribution.

Introduction

The regulation of transcription is an important topic of modern biology. Apart from molecular factors binding directly to DNA and changes in the local chromatin environment, the nuclear organization of chromatin has come into focus as a potential level of genome regulation (for reviews see Kosak and Groudine 2004; Foster and Bridger 2005; Cremer et al. 2006; Fraser and Bickmore 2007; Lanctôt et al. 2007; Meaburn and Misteli 2007; Misteli 2007). To understand this level of genome organization, it is essential to know the three-dimensional distribution of the genome. During interphase, chromosomes occupy a restricted space in the cell nucleus, the chromosome territory (Cremer et al. 2001, 2006; Meaburn and Misteli 2007). A nonrandom distribution of chromosome territories was described in nuclei of human origin (Croft et al. 1999; Cremer et al. 2001; Bolzer et al. 2005; Wiblin et al. 2005) from other primates (Tanabe et al. 2002; Neusser et al. 2007), mice (Parada et al. 2004; Mayer et al. 2005), and chicken (Habermann et al. 2001; Stadler et al. 2004). While in spherical lymphocyte nuclei of humans and other primates, a sorting of gene-dense chromosome territories towards the center and of gene-poor ones towards the nuclear periphery was the dominant pattern; findings in fibroblast nuclei (Croft et al. 1999; Boyle et al. 2001; Cremer et al. 2001) were more difficult to interpret. It now appears that in these flat, ellipsoid nuclei large chromo-

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somes are preferentially near the perimeter while small chromosomes are preferentially in a central cylinder and, in addition, gene-poor chromosome territories are in closer contact with the nuclear membrane than gene-rich ones (Bolzer et al. 2005). In chicken, small chromosomes are gene rich and were found centrally while large chromosomes are gene poor and were found peripherally (Habermann et al. 2001; Stadler et al. 2004).

Mouse chromosomes show comparatively little variation in size (195–61 Mbp compared to 245–47 Mbp in humans) and gene density (15.9–7.5 genes per megabase pair compared to 23–3.5 genes per megabase pair in humans; all values disregarding Y chromosomes (Ensembl Genome Browser, <http://www.ensembl.org>)). This suggested the possibility of a less pronounced nuclear sorting. In spite of the smaller differences, however, we previously showed that gene density as well as size-dependent sorting of chromosome territories occurs in six investigated cell types, embryonic stem (ES) cells, macrophages, lymphocytes, fibroblasts, myoblasts, and myotubes (Mayer et al. 2005). We and others (Parada et al. 2004; Wiblin et al. 2005) also described significant differences in chromosomal distribution between cell types. The reasons for these differences remain unclear; an obvious candidate is expression-related positioning in combination with cell-type-specific expression behavior. Studies investigating stably integrated transgenes in cultured hamster and mouse cells described cases where nuclear positioning was indeed expression dependent, with expressed loci more internal than inactive ones (Tumbar and Belmont 2001; Dietzel et al. 2004; Chuang et al. 2006). Nuclear positioning can be influenced by expression also for endogenous human or mouse gene loci, a fact maybe best illuminated by genes with monoallelic expression, where both genes show a different topology (Dietzel et al. 1999; Takizawa et al. 2008). Cases of gene repositioning upon transcriptional activation have been documented relative to their chromosome territory (Volpi et al. 2000; Williams et al. 2002; Chambeyron and Bickmore 2004), relative to centromeric heterochromatin (Brown et al. 1997), for movement away from peripheral heterochromatin (Kosak et al. 2002; Zink et al. 2004; Ragoczy et al. 2006; Williams et al. 2006) and for other movements towards more central areas (Takizawa et al. 2008). However, expression has also been reported for genes at the nuclear periphery (Nielsen et al. 2002; Ragoczy et al. 2006) and a study using a transgene tethered to the nuclear lamina could show that this transgene maintains transcriptional activity (Kumaran and Spector 2008). Two studies on a number of endogenous human genes could not find a relationship between expression state and radial nuclear positioning (Murmman et al. 2005; Küpper et al. 2007). It therefore appears that a positioning of transcribed regions towards the nuclear center is not a

requirement for all genes. These two studies instead described a correlation of the radial position of a gene locus and the local gene density within a 2- or 10-Mbp genomic window around the locus. In addition, one of these studies compared radial distribution of pools of gene loci with GC content and found a correlation of high GC percentages and internal positioning (Küpper et al. 2007).

In the current study, we attempted to discriminate between local gene density and other factors in the nuclear positioning of endogenous gene loci, such as GC content. We investigated gene loci in cycling mouse ES cells and in postmitotic in vitro differentiated mouse macrophages. Investigated gene loci included cases of similar gene density but different GC content. By investigating individual loci and not pools of loci, we avoided potential dilution of positioning effects due to averaging. Our results show that the preferential nuclear distribution of a given mouse gene locus can differ from cell type to cell type. In most cases, the average nuclear radial position of a locus was significantly different from the average radial position of the harboring chromosome territory, indicating a preference of the gene locus for specific subregions of the territory. Our dataset did not show a correlation of radial nuclear gene locus positioning with local gene density as it was described in human cells (Murmman et al. 2005; Küpper et al. 2007). Instead, we found a correlation with GC content and with interspersed repetitive elements.

Materials and methods

Cells

Mouse ES cells of the male CCE line were kindly provided by C. Bonifer, Leeds, UK and cultured in gelatinized (0.1% in water for 1 h) flasks on a feeder layer of mouse embryonic fibroblasts (MEF) in Dulbecco modified Eagle's minimal essential medium with 15% fetal calf serum (tested for ES cells) and leukemia inhibitory factor (Faust et al. 1997). MEFs from 13.5-day embryos were kindly provided by Christian Lanctôt, Munich, Germany and inactivated with mitomycin C (10 µg/ml for 2.5 h at 37°C). One hundred thousand feeder cells per square centimeter were plated in gelatinized flasks at least 1 day before seeding ES cells. The undifferentiated state of ES cells was confirmed in control experiments by staining for oct3/4 and alkaline phosphatase, resulting regularly in at least 90% positive cells (Supplemental Fig. 1a,b). Differentiation of ES cells to macrophages was started by cocultivation of ES cells on OP9 stroma cells (Nakano et al. 1994) as described (Schroeder et al. 2003). At day 8 of differentiation, suspension cells were transferred to cell culture flasks using medium containing macrophage colony-stimulating

factor (M-CSF) and interleukin 3 (IL-3). These cytokines were obtained by cultivation of L-cells and X63 AG-653 cells (kindly provided by C. Bonifer), transgenically expressing and secreting M-CSF or IL-3, respectively (Faust et al. 1994). On day 12, the culture contained adherent macrophages. Cells were transferred onto glass coverslips and fixed the following day. Terminal differentiation to macrophages was tested by antibody staining for the surface antigen CD11b, cell shape and behavior (adherence), the absence of BrdU incorporation, and the uptake of fluorescent beads (Supplemental Fig. 1c–i). Differentiation efficiency to macrophages was close to 100%. Cell numbers were small, however. Pmi28 primary mouse myoblasts were kindly provided by A. Starzinski-Powitz (Kaufmann et al. 1999) and cultured as described (Cardoso et al. 1997). For microscopic preparations, glass coverslips with a thickness of 170 μm were used (Karl Hecht KG, Sondheim/Rhön, Germany). For ES cells, they were coated with gelatine and cell suspension was incubated for 1–2 h to allow attachment. 3D fixation was performed with 4% formaldehyde freshly made from paraformaldehyde (Dernburg and Sedat 1998), buffered in 0.75 \times phosphate-buffered saline (PBS; ES cells) or 1 \times PBS for 10 min after a short wash in the respective PBS buffer. Permeabilization was performed as described elsewhere (Hepperger et al. 2007). Slides were kept at 4°C in 50% formamid/2 \times SSC until hybridization. Air drying was carefully avoided during all steps.

DNA probes and FISH

Mouse chromosome paint probes produced by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) from sorted chromosomes (Rabbitts et al. 1995) were kindly provided by N. Carter, Cambridge, UK or J. Wienberg, Chrombiosa, Raubling, Germany. Bacterial artificial chromosome (BACs) were ordered from BAC-PAC resource center (Oakland, CA, USA, <http://bacpac.chori.org>) and tested for correct chromosomal location by fluorescence in situ hybridization (FISH) together with the respective library on metaphase spreads. In our experience, about 10% of BACs hybridize on wrong or additional chromosomes. The following BACs were used: RP23-326O5 (containing lysozyme), RP23-47G13 (myb), RP24-79I7 (β -globin), RP23-368L18 (CD11b=Itgam), RP23-75C13 (oct3/4=pou5f1), RP24-376D9 (mpo), RP23-95B6 (β -actin), and RP23-301A23 (albumin). Where possible, sequence identity of selected BACs was additionally confirmed by PCR with primers designed for reverse transcription (RT)-PCR experiments. This was the case for BACs from the following loci: lysozyme, β -globin, CD11b, oct3/4, β -actin, albumin, and Beckwith–Wiedeman syndrome (BWS) locus (Hepperger et al.

2007). Labeling of chromosome paints was by DOP-PCR using dinitrophenol (DNP)-deoxyuridine triphosphate (dUTP). BACs were labeled with digoxigenin-dUTP or biotin-dUTP by nick translation. Ten microliter hybridization mix (50% formamide, pH=7, 10% dextran sulphate in 1 \times SSC) contained DNA from 10 μl chromosome paint DOP-PCR product, 200 ng of each BAC plus 40 μg mouse C₀t1-DNA (Invitrogen). Simultaneous DNA denaturation was performed at 75°C for 2–3 min. Hybridization was at 37°C for 2–3 days; detection was as described (Hepperger et al. 2007) using the following antibodies: Rabbit α DNP (Sigma, Deisenhofen, Germany), goat α rabbit Cy3 (Amersham-Pharmacia Biotech, Braunschweig, Germany), avidin Alexa 488, goat α rabbit Alexa 488 (both from Molecular Probes, Eugene, OR, USA), goat α avidin fluorescein isothiocyanate, goat α streptavidin Bio (both from Vector Laboratories, Burlingame, CA, USA), mouse α Dig Cy5, mouse α Dig Cy3 (both from Jackson ImmunoResearch, West Grove, PA, USA), goat α mouse Cy5, sheep α mouse Cy3 (both from Dianova, Hamburg, Germany), and streptavidin Cy5 (FMC Rockland, ME, USA). 4',6-Diamidino-2-phenylindole (DAPI) or TO-PRO-3 (Molecular Probes) was used as DNA counterstain. The splicing factor SF2/ASF was detected with a mouse antibody (Zymed Laboratories, South San Francisco, CA, USA, cat # 32-4600).

Confocal microscopy, image evaluation, and statistics

Confocal image stacks were generated on Leica TCS SP2 and Zeiss LSM 410 microscopes with voxel sizes of 80 nm in xy and 250 nm in z or less. The software ImageJ (<http://rsb.info.nih.gov/ij/>) was used on confocal image stacks for shift correction of chromatic aberration, processing where necessary (e.g., erasing neighboring nuclei), and determination of signal thresholds for subsequent computerized image analysis. To achieve a minimum of variability introduced by personal bias, all thresholds were determined on single color image stacks by the same individual. When subsets of data were reevaluated by another person or the same person on a different day, similar results were obtained. Absolute 3D distance measurements from signal voxels to the nearest surface of the nucleus or chromosome territory was performed with the software eADS, developed by J. von Hase and T. Thormeyer (Albiez et al. 2006; Küpper et al. 2007). Briefly, at the determined threshold, a surface around the reference object (nucleus or territory) is generated. Thereafter, for each voxel of a segmented signal, the shortest 3D distance to the surface is calculated. Intensity-weighted frequencies were plotted in classes with 250-nm width. Signals inside the reference object obtain negative values, those outside positive values. *p* values for a pairwise comparison of the radial nuclear distributions

within a population of nuclei were calculated with the Wilcoxon signed rank test (Sigma Stat 3.0, SPSS Inc., Chicago, IL, USA.) which performs a direct comparison of the values from each nucleus. Therefore, this test may find significant differences between populations which may be obscured when pooled values are investigated. To this end, for each nucleus, the radial distribution of a given type of signal (gene or territory) was represented by the intensity-weighted mean value of the signal-to-surface distance. The average overall investigated nuclei was used for correlation analysis. Pearson correlation coefficients were calculated in Microsoft Excel 2003.

To allow a comparison of gene locus distributions in different cell types despite different nuclear shapes, signal distributions were normalized by the DNA distribution. If at a given distance to the nuclear surface either the DNA content or the gene locus signal content was less than 4% of the nuclear signal, values were excluded from diagrams and further calculations, to avoid artificially large ratios due to chance results for small absolute values. Depending on the width of the distribution, we thus obtained between four and nine normalized data points per gene locus and cell type. A statistical comparison between such normalized gene locus distributions in different cell types was performed in three steps. First, we fitted a curve with the regression wizard of Sigma Plot 10.0 (SPSS Inc.), using a quadratic polynomial. For further calculations, the fitted curve was selected which showed the best correlation with its respective experimental data points. Respective R^2 values were as follows: albumin in ES cells 0.99, β -actin in myoblasts 0.98, β -globin in macrophages (M) 0.99, CD11b in M 0.91, lysozyme in M 0.89, myb in ES 0.84, oct3/4 in ES 0.91, and mpo in M 0.95. Second, for each experimental data point, the squared difference to the respective value of the fitted curve was calculated. However, for any given gene locus distribution, this was limited to the distance-to-nuclear-surface range that was covered in both (or all three for β -actin, albumin, oct3/4) cell types. Third, the squared differences were compared statistically (Sigma Stat). When distributions from two cell types were investigated, the Mann Whitney rank sum test was used. When three cell types were investigated, a Kruskal–Wallis one-way analysis of variance was applied.

RT-PCR

Exon sequences were copied from <http://www.ensembl.org>. RT-PCR primers with a length between 20 and 25 bases and $T(m)$ between 55°C and 66°C were designed with the software FastPCR (<http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm>) such that the forward primer was within one of the last two exons and the reverse primer was in the 3' untranslated region, resulting in a PCR product

length of about 200 to 800 bp. Primers were ordered from MWG-Biotech, Ebersberg, Germany. A complete list with sequences and expected lengths of cDNA and genomic DNA fragments is given in Supplementary Table 1. Cellular RNA was isolated with peqGold (peqLab, Erlangen, Germany) according to the manufacturer's instructions. In each reaction, cDNA was generated from 5 μ g RNA with Super Script II Reverse Transcriptase (Invitrogen). Of the total reaction volume (20 μ l), 1/10 was used as undiluted solution for gel analysis.

Databases and genomic properties

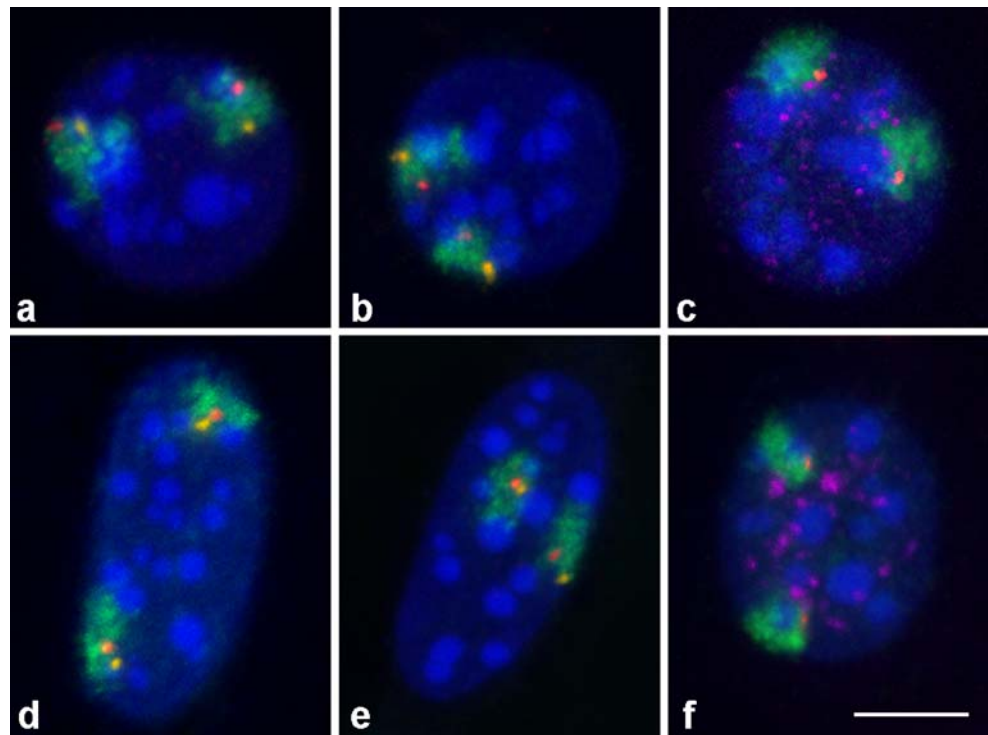
Suitable BACs as well as neighboring genes were identified in the "cytoview" display of the Ensembl Genome Browser (<http://www.ensembl.org>). To identify the 2-Mbp window for each BAC, the central base was determined from the borders of the region shown in the NCBI Map viewer developed at the National Center for Biotechnology information (<http://www.ncbi.nlm.nih.gov/Genomes>, Build 37.1 from July 2007) after searching for the BAC name. The points 1 or 5 Mbp downstream and upstream were calculated and used as new region borders. For the new region, the numbers of genes and CpG islands were copied and the sequence was downloaded. Obtained sequences were submitted to RepeatMasker (version open-3.1.6, http://www.repeat_masker.org) to obtain the percentage consisting of GCs and of repetitive elements.

Results and discussion

Experimental design

We investigated the spatial nuclear distribution of individual gene loci in cycling mouse embryonic stem cells and in postmitotic macrophages differentiated in vitro from the ES cells (Supplemental Fig. 1). Nonconfluently growing ES cells have spherical nuclei. Macrophages investigated here have round to oval nuclei with a height about half the average diameter in xy (Mayer et al. 2005 and data not shown). We labeled the position of gene loci together with their respective chromosome territories by multicolor FISH on cells fixed with buffered formaldehyde (Fig. 1). This procedure has been shown previously to structurally preserve large-scale chromatin structure at the resolution of light microscopy (Solovei et al. 2002; Hepperger et al. 2007; Kim et al. 2007). In total, over 400 morphologically preserved nuclei with multicolor labeled gene loci and chromosome territories were recorded by confocal microscopy as multicolor 3D stacks and evaluated by computational 3D image analysis. For all FISH signals, distances to the nuclear border and to the surface of the respective

Fig. 1 FISH on structurally preserved nuclei. **a–c** Mouse embryonic stem cells. **d–f** In vitro differentiated macrophages. **a, d** FISH with probes for mouse chromosome (MMU) 5 (green) and BACs for the β -actin (yellow) and the albumin locus (red). **b, e** MMU7 (green), β -globin (yellow), and CD11b (red) loci. **c, f** MMU17 (green), oct3/4 locus (red), and antibody staining against the splicing factor SF2/ASF (purple). Projections of confocal image stacks are shown. DNA counterstain (DAPI) in blue. Scale bar 5 μ m



chromosome territory were determined. We selected pairs of loci from three different chromosomes: the β -actin locus and the albumin locus from *Mus musculus* chromosome 5 (MMU5); MMU7 with β -globin and CD11b and MMU10 with myb and lysozyme. In additional experiments, the oct3/4 locus (also known as oct3, oct4, and Pou5f1) on MMU17 and the myeloperoxidase (mpo) locus on MMU11 were detected. To allow distinguishing gene-specific relocation events from repositioning of whole chromosome territories, all gene loci were detected together with their harboring chromosomes. In a previous study, we determined the 3D positioning of the mouse region homologous to the human Beckwith-Wiedeman-Syndrome region in ES cells (Hepperger et al. 2007) and included it in the current comparison. Supplemental Fig. 2 shows the chromosomal location of the loci as well as the immediate genomic neighborhood of each locus, with neighboring genes.

The radial nuclear distribution of gene loci can differ significantly from the distribution of their harboring chromosome territory

While all gene loci were found in or close to their respective chromosome territory (see below), the distribution of distances to the nuclear surface differed in most cases significantly for the gene locus and the harboring chromosome territory (Fig. 2, Table 1). Thus, the distribution of genes within a chromosome territory is not random. For example, the β -globin locus in ES cells is significantly

closer to the nuclear surface than the average of the harboring MMU7 (Fig. 2c, Table 1). In contrast, the CD11b locus on the same chromosome is much more internal than both the β -globin locus and the average of the MMU7 territory, showing that two gene loci on the same mouse chromosome can also differ significantly from each other in their preferred radial nuclear position (all three p values < 0.001). Significant differences (Table 1) between the nuclear radial distribution of genes and their harboring territory in ES cells were also found for the albumin (Fig. 2a) and oct3/4 (Fig. 2i) genes and the BWS region (Hepperger et al. 2007), in macrophages for the actin (Fig. 2b), β -globin, CD11b (Fig. 2d), mpo (Fig. 2h), and oct3/4 genes (Fig. 2j) and in myoblasts for the actin gene (Fig. 2l; see Table 1 for p values). Our results thus indicate a preference of gene loci for a particular subregion of the chromosome territory while the territory has itself a preferred radial nuclear position. This argues for a polar radial organization of chromosome territories, as has been suggested by others (Ferreira et al. 1997; Croft et al. 1999; Sadoni et al. 1999; Saccone et al. 2002; Scheuermann et al. 2004; Küpper et al. 2007).

Radial positioning was not deterministic. All investigated chromosome territories and most investigated gene loci could be found sometimes at the nuclear border and sometimes up to 3 μ m in ES cells or 2 μ m in macrophages away from the nuclear border. Exceptions were the BWS homologous region in ES cells (Hepperger et al. 2007), the CD11b locus in macrophages (Fig. 2d), and the oct3/4 gene

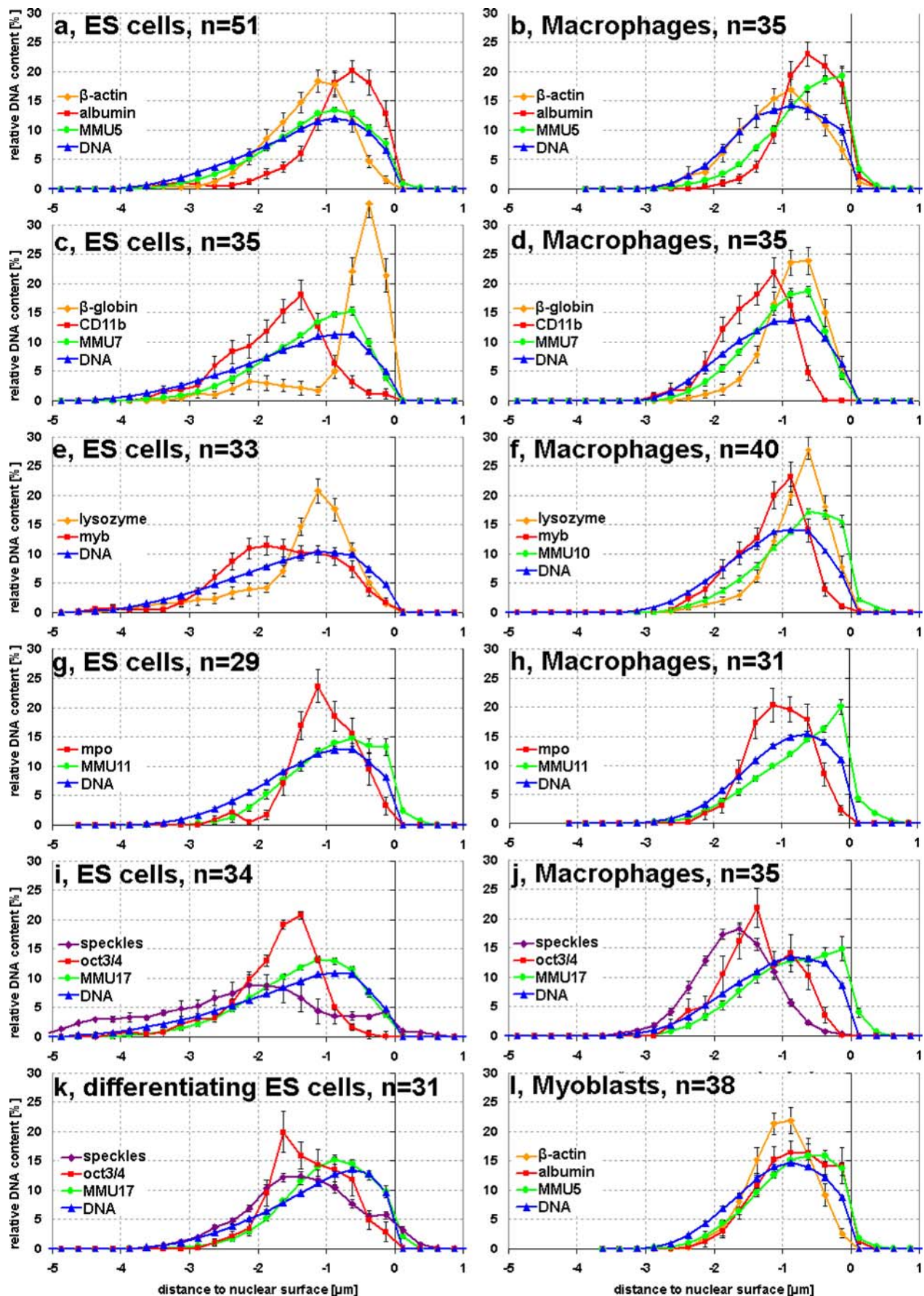


Fig. 2 a–l Absolute distance of gene loci to the surface of nuclei. Values on the x-axis show the distance to the nuclear surface; negative values represent signals inside the nucleus. Segmented nuclei were divided in shells of 0.25- μ m thickness. For each nucleus, the percentage of given FISH signals or DNA counterstain in each shell was calculated. Curves show the average overall nuclei; respective numbers are given above each graph. The standard error of the mean is indicated by error bars. The respective chromosome territory is always in green, the DNA counterstain in blue. In nuclei stained for MMU17 and oct3/4, the splicing factor SF2/ASF was codetected by antibody staining. Macrophage and myoblast nuclei are flatter than investigated ES cell nuclei. As a consequence, most FISH signals are closer to the nearest surface. Note the corresponding shift of most curves to the right, representing values closer to the nuclear surface. The combination of MMU17, oct3/4, and speckles was detected also in “differentiated ES cells,” grown in medium without inhibitors of differentiation for 7 days. Corresponding nuclei are also flatter than in undifferentiated ES cells

in both cell types (Fig. 2i,j), which were not found within the outermost 0.25 μ m of the counterstained nucleus.

The β -globin locus is positioned at the nuclear periphery

The β -globin locus has been intensely investigated in earlier studies of nuclear organization (e.g. Brown et al. 2001, 2006; Francastel et al. 2001; Ragoczy et al. 2003, 2006); it is thus worthwhile to compare its distribution in our 3D datasets with previously available data. In a study on human activated primary T cells, 90% of the inactive β -globin genes were found in association with centromeric heterochromatin (Brown et al. 2001). During murine erythroid maturation, a movement from the nuclear periphery to more central regions was observed (Ragoczy et al. 2006). Francastel et al. (2001) described a tethering of the unexpressed β -globin locus to chromocenters in mouse erythroleukemia (MEL) cells while another study from the

same group could not confirm this (Ragoczy et al. 2003). A transgene array in MEL cells with regulatory elements from the β -globin locus did not colocalize with chromocenters (Dietzel et al. 2004). In structurally preserved mouse cell nuclei, centromeric heterochromatin is easily visualized by the bright DAPI staining of the chromocenters (Mayer et al. 2005). Of the 70 β -globin gene alleles in 35 three-dimensionally recorded macrophage nuclei recorded in this study, we found only ten touching a chromocenter, compared to eight of 70 alleles of CD11b, a gene for a surface antigen which is expressed in these cells (determined by antibody staining, Supplemental Fig. 1c,e–g). In ES cells, chromocenter touching fractions were 8/70 for both β -globin and CD11b. We thus did not observe an above-random tethering of the β -globin locus to chromocenters in either cell type. A pronounced positioning of the silent β -globin locus to centromeric heterochromatin may thus be a feature of specific cell types. However, our data did reveal a pronounced positioning of the β -globin gene locus at the nuclear periphery, in particular in ES cells (Fig. 2c,d). Here, three quarters of its total signal intensity was within 0.75 μ m of the border of the segmented nucleus (Fig. 2c). Visual inspection confirmed that the locus was often embedded in peripheral heterochromatin (Fig. 1b). Since the β -globin gene was also found at the nuclear periphery in immature murine erythroid differentiation stages from fetal liver (Ragoczy et al. 2006), it is possible that this locus is at the nuclear periphery in many nontumor cell types, except when activated.

Gene locus positioning is cell type specific

In an earlier study, we showed that the preferred radial nuclear position of mouse chromosome territories can

Table 1 Statistical comparison of absolute distances to the nuclear surface

	Gene1–gene2	Gene1–terr	Gene2–terr	Gene1–DNA	Gene2–DNA	terr–DNA
ES: act/alb	<0.001	0.112	<0.001	0.047	<0.001	<0.001
ES: β -glob/ CD11b	<0.001	<0.001	<0.001	<0.001	0.021	<0.001
ES: lys/ myb	0.006	–	–	0.037	0.26	–
ES: mpo	–	0.066	–	0.008	–	<0.001
ES: BWS	–	<0.001	–	0.65	–	<0.001
ES: oct3/4	–	<0.001	–	0.161	–	<0.001
Diff. ES: oct3/4	–	<0.001	–	0.167	–	0.001
Mph: act/alb	<0.001	<0.001	0.107	0.55	<0.001	<0.001
Mph: β -glob/ CD11b	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mph: lys/ myb	<0.001	0.952	<0.001	<0.001	0.962	<0.001
Mph: mpo	–	<0.001	–	0.342	–	<0.001
Mph: oct3/4	–	<0.001	–	0.003	–	0.001
Myoblasts: act/alb	0.01	0.003	0.817	0.425	<0.001	<0.001

p values were calculated for all comparisons possible within a given population of nuclei and are based on a pairwise comparison of the mean signal positions in each nucleus. *Diff.* ES differentiating ES cells, *Mph* macrophages.

change with cell type (Mayer et al. 2005). The current data demonstrate that the same is true for some individual gene loci. This can be visualized best when the amount of gene signal at a certain distance from the nuclear surface is normalized by the DNA content at that distance (Fig. 3). Such a normalization accommodates for nuclear shape differences which lead to a higher percentage of nuclear volume near the nuclear surface in oblate macrophages compared to spherical ES cell nuclei (compare DNA

distribution in Fig. 2b,d,f,h,j with Fig. 2a,c,e,g,i). Distributions of three of the eight loci (CD11b, oct3/4, and mpo, $p > 0.06$) did not reveal significant differences between the investigated cell types. However, a strongly cell-type-dependent radial nuclear positioning became obvious for the β -globin gene locus (Fig. 3c), which is more internal in macrophages than in ES cells ($p = 0.029$) and the lysozyme ($p = 0.032$) and myb ($p = 0.002$) gene loci (Fig. 3d), which are more internal in ES cells.

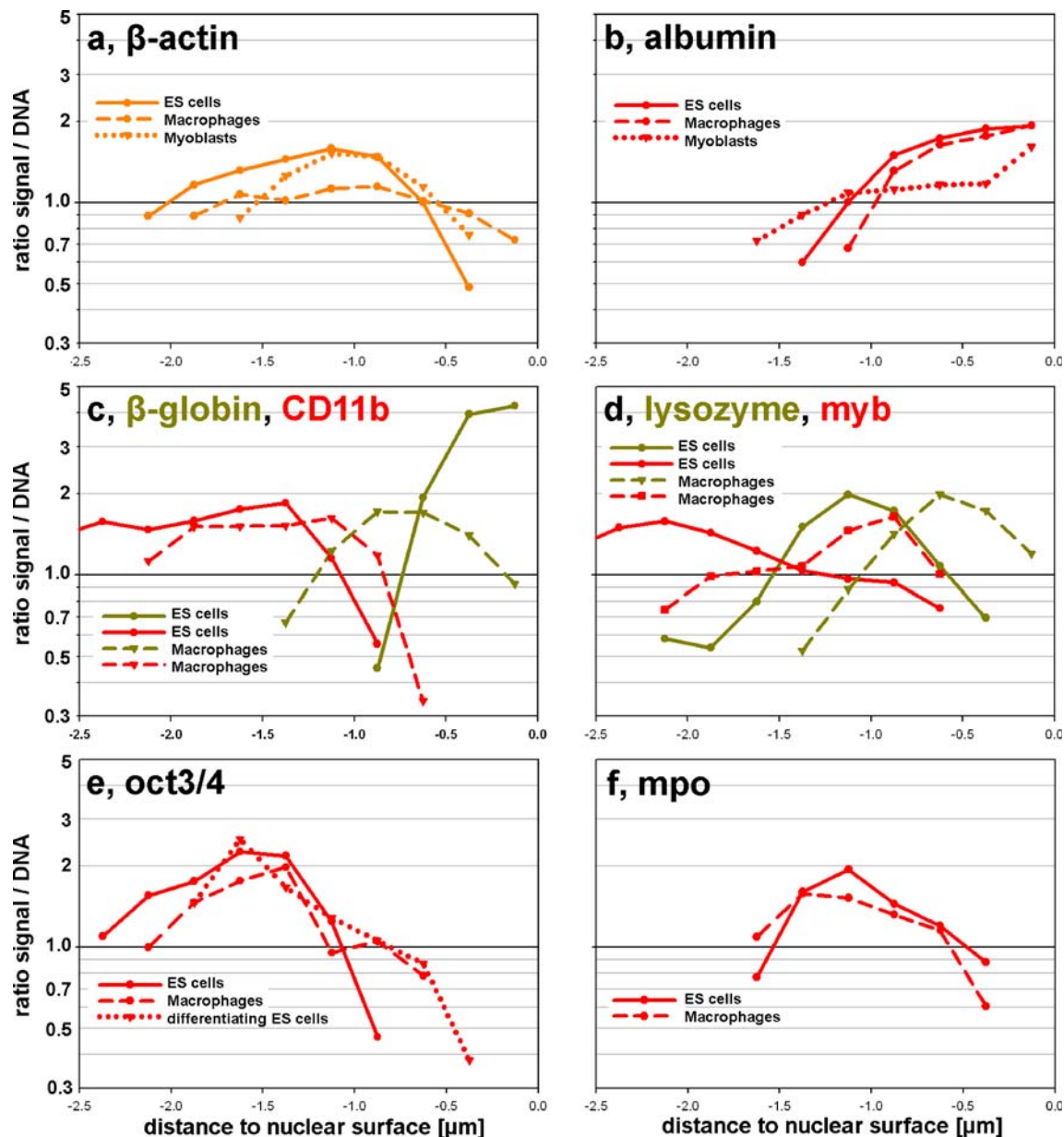


Fig. 3 a–f Distance of gene loci to the surface of nuclei normalized by DNA content. The percentage of a gene signal at a given distance to the nuclear surface (see Fig. 2) was normalized by division through the DNA content at the same distance. To avoid chance results with small numbers, results were calculated only if the percentage of the gene signal and the DNA at the given distance to the nuclear surface

were both above 4% of the total signal. Curves for a given gene in different cell types are shown in the same graph. For example in a the β -actin gene is distributed rather similar to nuclear counterstain in macrophages but not in ES cells and myoblasts, arguing for a cell-type-specific distribution ($p = 0.01$). See main text for discussion of the other panels

Significant differences for the distributions in ES cells, macrophages, and myoblasts were also detected for the β -actin locus ($p=0.01$; Fig. 3a) and the albumin locus ($p=0.024$; Fig. 3b). In addition, in ES cells, the albumin locus was much closer to the nuclear border than the average of the harboring MMU5 (Fig. 2a, $p<0.001$), while in macrophages (Fig. 2b, $p=0.1$) and in myoblasts (Fig. 2l, $p=0.8$) there was no significant difference. Thus, only in ES cells but not in the other two tested cell types the albumin locus preferentially occupied a subregion of the territory oriented towards the nuclear border, providing an example in addition to the β -globin locus of cell-type-specific localization to particular subregions of the chromosome territory.

Taken together, our observations suggest that cell-type-specific positioning of gene loci is not a simple consequence of a relocation of the harboring chromosome territory as a whole. We conclude that intrachromosomal rearrangements are required to explain our results. Cell-type-specific radial nuclear distributions of chromosome territories and gene loci argue against a regulation of the radial position exclusively by genomic features, such as local gene density.

Radial nuclear positioning of gene loci does not correlate with local gene density

In a previous study, we showed for mouse chromosome territories a positive correlation between gene density and a more internal position in the nucleus (Mayer et al. 2005). Others have described such a correlation for chromosome territories of humans and other primates (see “Introduction”). Recent studies on human cells found a correlation of the radial nuclear position of gene loci with local gene density, i.e., with the number of genes present in a 2- or 10-Mbp window around the locus (Murmman et al. 2005; Küpper et al. 2007). However, in our current data, we do not find such a correlation. For each gene locus, we calculated the average radial nuclear position which we compared to the gene density in a 2- and 10-Mbp window around the locus (Tables 2, 3, Fig. 4b). The Pearson correlation coefficient R becomes 1 or -1 for a perfect positive or negative correlation, respectively, and 0 if no correlation exists. Neither in mouse ES cells ($R=-0.05$ for 2 Mbp, $R=-0.15$ for 10 Mbp) nor in mouse macrophages ($R=0.27$ and 0.08) did we find a good correlation between locus position and gene density.

Nuclear positioning correlates well with GC content and the presence of certain repetitive elements

In general, a good correlation between gene density and high GC content can be expected (Bernardi 2000; Waterston et al. 2002). For complete mouse chromosomes, this is

indeed the case ($R=0.85$ for autosomes). For the set of gene loci investigated in the current study, however, there was no correlation between gene density and GC content ($R=-0.02$ and 0.26 for 2- and 10-Mbp windows, respectively; Fig. 4a). Instead, we included GC-poor but gene-rich and GC-rich but gene-poor cases. Tables 2 and 3 list sequence properties of the 2- and 10-Mbp windows around the investigated loci. Thus, the set of selected loci allowed us to distinguish relations of nuclear positioning with gene density from relations with GC content. In addition, we investigated correlation with the density of CpG islands, short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs), and long terminal repeats (LTRs). Visual representation of these properties in the 2-Mbp windows around the investigated loci (Supplemental Fig. 3) shows that these properties are evenly distributed in these windows.

In ES cells and in macrophages, a more internal nuclear position of gene loci correlated positively with high GC content ($R=0.61$ and 0.63 , respectively; Fig. 4c) and a high frequency of LTR elements ($R=0.66$ and 0.61 , Fig. 4e) in the 2-Mbp window. The presence of LINEs correlated negatively with an internal position, in particular in ES cells ($R=-0.65$ and -0.49 , Fig. 4d). Correlation with the presence of CpG islands and SINEs was still higher than for gene density (see Table 4 for all R values). In general, we observed a better correlation of nuclear positioning with these genomic properties in 2-Mbp windows than in 10-Mbp windows, suggesting that certain properties related to positioning are diluted when the larger window is used for analysis.

Since 3D FISH is not a high-throughput technique, the number of gene loci investigated in such studies is necessarily small compared to the number of genes in the genome. It is thus possible that any finding in such a study is due to a particular selection of gene loci with exceptional behavior and not representative for the genome on the whole. Our results invite to speculate, however, that gene-density-related positioning of chromosomal loci and chromosomes as observed in previous studies may be a side effect of positioning driven by other properties.

Gene positioning and expression

To investigate the relation between nuclear positioning and transcription, we determined expression of genes in ES cells by RT-PCR. In macrophages, this was not possible due to the small amount of cells obtained by in vitro differentiation. Published studies relating nuclear positioning to expression often investigated the expression of only one gene of interest. However, this leaves the possibility open that neighboring genes with different regulation patterns obscure the findings. We therefore decided to also

Table 2 Characteristics of investigated gene loci, 2-Mbp windows

	β -actin	Albumin	myb	lys	β -globin	CD11b	oct3/4	BWS	mpo
Dist. to surface ES cells [μ m]	1.3	0.8	1.7	1.4	0.7	1.7	1.7	1.5	1
Dist. to surface Macrophages [μ m]	1.04	0.62	1.2	0.8	0.8	1.4	1.3		1
Genes/Mbp	25.6	13	12	17.5	69.5	38	73	16.5	19.5
CpG islands/ Mbp	181.1	60	90	194	44.5	153.5	197.5	66.5	144.9
GC content	48.31	40.60	42.41	44.18	39.13	45.89	48.12	47.81	45.78
SINEs:	21.36	6.05	7.5	12.08	3.54	16.65	14.13	8.82	20.16
Alu/B1	7.19	2.06	2.88	4.09	1.42	6.78	5.99	2.81	8.29
B2-B4	13.28	3.35	3.94	7.01	1.91	8.89	7.78	5.54	10.41
IDs	0.57	0.07	0.23	0.28	0.05	0.29	0.18	0.15	0.47
MIRs	0.31	0.57	0.45	0.68	0.15	0.69	0.18	0.31	1
LINEs:	3.31	17.36	9.47	6.18	33.13	7.98	5.69	7.16	3.66
LINE1	3.1	16.82	9.02	5.31	33.03	7.49	5.47	7	3.04
LINE2	0.19	0.49	0.42	0.83	0.08	0.42	0.19	0.14	0.5
L3/CR1	0.01	0.04	0.03	0.02	0.01	0.04	0.02	0.02	0.09
LTR elements:	11.11	8.36	9.11	12.51	10.04	15.17	16.73	11.49	7.11
MaLRs	4.51	3.8	3.55	5.92	1.95	4.57	3.05	4.63	2.97
ERV L	1.5	0.65	0.91	1.62	1.17	2.16	2.01	1.46	0.74
ERV_classI	0.99	0.41	0.55	0.97	1.37	0.8	3.02	0.54	1.04
ERV_classII	4.11	3.48	4.07	4	5.56	7.64	8.64	4.87	2.35

The first two lines show the average mean distance of the respective locus to the nuclear surface in micrometer in ES cells and macrophages, respectively. The two following lines give genes per megabase pair and CpG islands per megabase pair, factoring in that due to gaps in the current genome built, for the β -actin and mpo loci, only 1.8 Mbp of sequence are available in this region (see Supplemental Fig. 3). All subsequent lines give the percentage by which the sequence in the given window is composed of the respective element.

determine expression of other genes included on the BAC or in the neighborhood. Indeed, we detected mRNA from at least one gene, including cases where the original target gene was silent, for all regions investigated, except for the β -globin locus (Supplemental Fig. 4). We conclude that the expression pattern of a single gene does not provide

sufficient information for a correlation with nuclear positioning. Expression levels were low for genes at the albumin locus while β -actin, CD11b, myb, lysozyme, and oct3/4 loci contained at least one strongly expressed gene.

We thus found that, with the albumin and the β -globin loci, those two gene regions with no or low-level

Table 3 Genomic properties of 10-Mbp windows around investigated loci

	β -actin	Albumin	myb	lys	β -globin	CD11b	oct3.4	BWS	mpo
Genes/Mbp	20.6	17.4	11.1	9.8	41.4	18.8	38.9	31.1	14.1
CpG islands/ Mbp	148.2	59.2	73.1	124.8	60	91.9	111.9	84.4	85.8
GC content	46.87	40.57	41.36	41.93	42.13	45.38	43.58	47.93	43.01
SINEs:	16.69	6.34	6.39	7.44	8.77	10.99	9.28	9.1	13.75
Alu/B1	5.13	2.34	2.14	2.29	3.17	3.86	3.63	3.35	5.12
B2-B4	10.81	3.53	3.64	4.41	4.65	6.03	5.32	5.36	7.57
IDs	0.38	0.1	0.14	0.16	0.19	0.23	0.15	0.15	0.29
MIRs	0.37	0.37	0.45	0.57	0.77	0.88	0.18	0.25	0.77
LINEs:	8.03	27.01	16.23	13.58	23.93	9.97	21.88	9.22	10.05
LINE1	7.73	26.59	15.8	12.91	23.23	9.37	21.69	9.06	9.46
LINE2	0.24	0.38	0.36	0.58	0.06	0.52	0.16	0.14	0.48
L3/CR1	0.03	0.03	0.06	0.06		0.05	0.02	0.01	0.07
LTR elements:	11.69	10.51	10.65	10.21	9.17	10.99	14.73	9.84	8.42
MaLRs	4.64	3.29	4.16	4.91	2.82	5.02	4.42	4.56	3.35
ERV L	1.42	0.89	1.05	1.46	1	1.61	1.29	1.25	0.97
ERV_classI	0.76	1.57	0.84	0.6	0.87	0.52	1.48	0.41	0.67
ERV_classII	4.88	4.74	4.59	3.23	4.47	3.82	7.54	3.62	3.42

Note that the BWS locus is less than 2 Mbp away from the telomere; its window thus contains only 6.6 Mbp. See legend to Table 2 for further details.

Fig. 4 Genomic properties of gene loci and their distance to the nuclear surface. **a** For the gene loci investigated in this study, GC content (y -axis) does not correlate with local gene density (x -axis). **b–e** The average distance of gene loci to the nuclear border (y -axis) in relation to **b** gene density, **c** GC content, **d** LINE content, and **e** LTR element content in a 2-Mbp window around the locus. Gene loci are indicated above or below the respective data points (β -gl = β -globin; act = β -actin). Values from ES cells are indicated by black dots (with a continuous regression line), those from macrophages by gray squares (with a dashed regression line). Since genomic properties do not change, values from the two cell types are always above each other. Data for the BWS homologous regions were taken from Hepperger et al. (2007) and were available for ES cells only. Since macrophages are flatter than spherical ES cells, in general, their distance to surface values is smaller (compare Fig. 2). For corresponding correlation coefficients, see Table 4. **f** The highest correlation found in this study ($R=0.83$) was between the average distance from a gene locus to the nuclear surface in ES cells (x -axis) to the respective average distance in macrophages (y -axis)

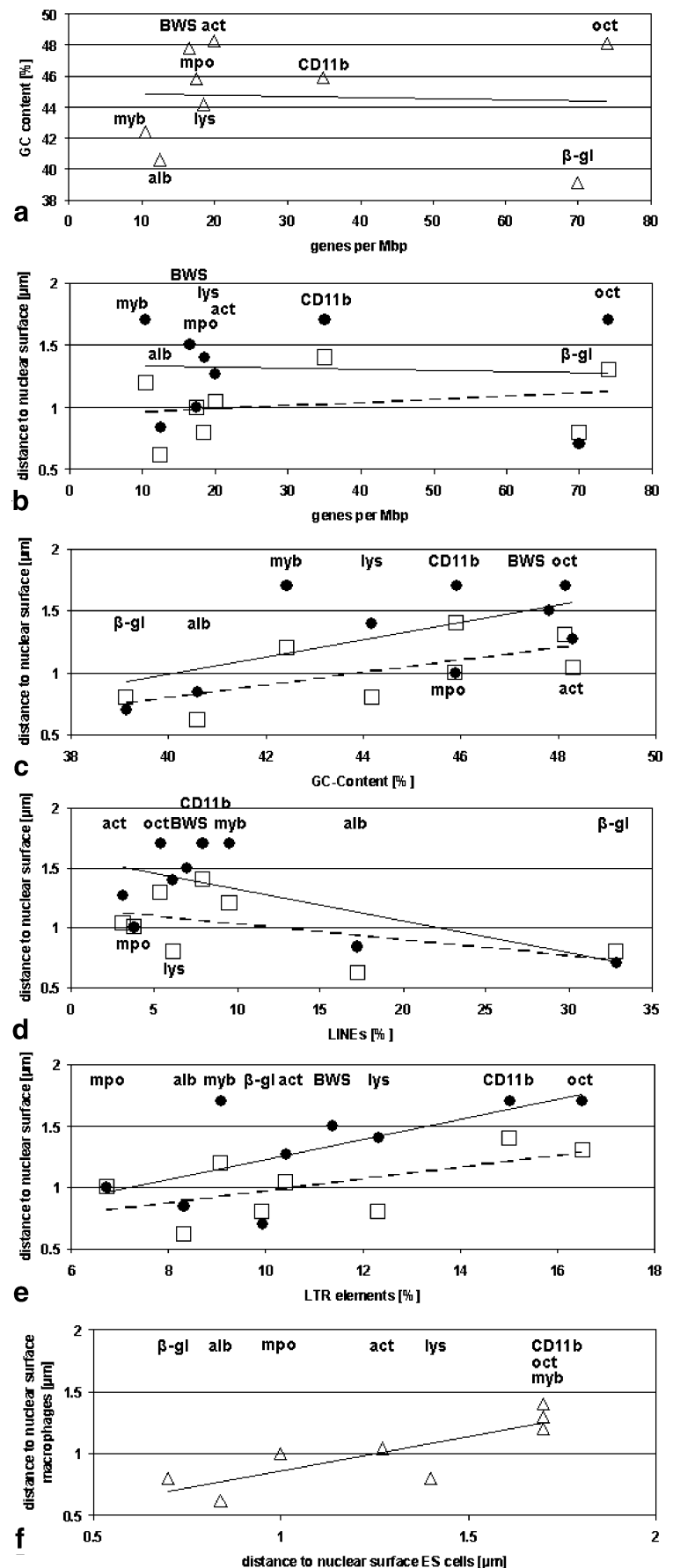


Table 4 Pearson correlation coefficients R and coefficient of determination R^2 for average gene locus positions and various genomic properties in the 2- and 10-Mbp windows (see Tables 2 and 3)

	2-Mbp window				10-Mbp window			
	ES cells: R	ES cells: R^2	Macrophages: R	Macrophages: R^2	ES cells: R	ES cells: R^2	Macrophages: R	Macrophages: R^2
ES to macrophages	0.83	0.68						
Genes per Mbp	−0.05	0.00	0.27	0.08	−0.15	0.02	0.08	0.01
CpG islands per Mbp	0.49	0.24	0.47	0.22	0.39	0.16	0.27	0.07
GC content	0.61	0.37	0.63	0.40	0.36	0.13	0.56	0.31
SINEs:	0.27	0.08	0.46	0.22	−0.06	0.00	0.27	0.07
Alu/B1	0.29	0.08	0.56	0.31	−0.09	0.01	0.32	0.10
B2-B4	0.27	0.08	0.41	0.17	−0.02	0.00	0.24	0.06
IDs	0.17	0.03	0.30	0.09	−0.10	0.01	0.23	0.05
MIRs	−0.07	0.00	−0.06	0.00	−0.29	0.08	0.03	0.00
LINEs:	−0.65	0.42	−0.49	0.24	−0.42	0.18	−0.46	0.21
LINE1	−0.64	0.41	−0.48	0.23	−0.41	0.17	−0.45	0.20
LINE2	0.07	0.01	−0.25	0.06	0.18	0.03	0.03	0.00
L3/CR1	−0.17	0.03	0.04	0.00	−0.10	0.01	−0.02	0.00
LTR elements	0.66	0.43	0.61	0.37	0.57	0.32	0.51	0.26
MaLRs	0.45	0.20	0.00	0.00	0.84	0.70	0.58	0.33
ERV_L	0.65	0.42	0.64	0.41	0.64	0.42	0.54	0.29
ERV_classI	0.20	0.04	0.37	0.14	−0.23	0.05	−0.25	0.06
ERV_classII	0.51	0.26	0.64	0.40	0.22	0.05	0.33	0.11

R^2 gives the proportion of the variance in the distribution which can be statistically explained by the investigated property. For example, 37% of the variance in the distribution of gene loci in ES cells can be statistically explained by the GC content.

transcription (Supplemental Fig. 4) are those which are the most peripheral in ES cell nuclei (Fig. 2a,c; compare Fig. 4f). This is consistent with previous data describing a more peripheral position for inactive than for active regions (see “Introduction”) but not with studies on human endogenous loci which could not find such a correlation (Murmman et al. 2005; Küpper et al. 2007; Meaburn and Misteli 2008). The reasons for these discrepancies remain unknown. They may involve differences between human and rodent nuclear organization.

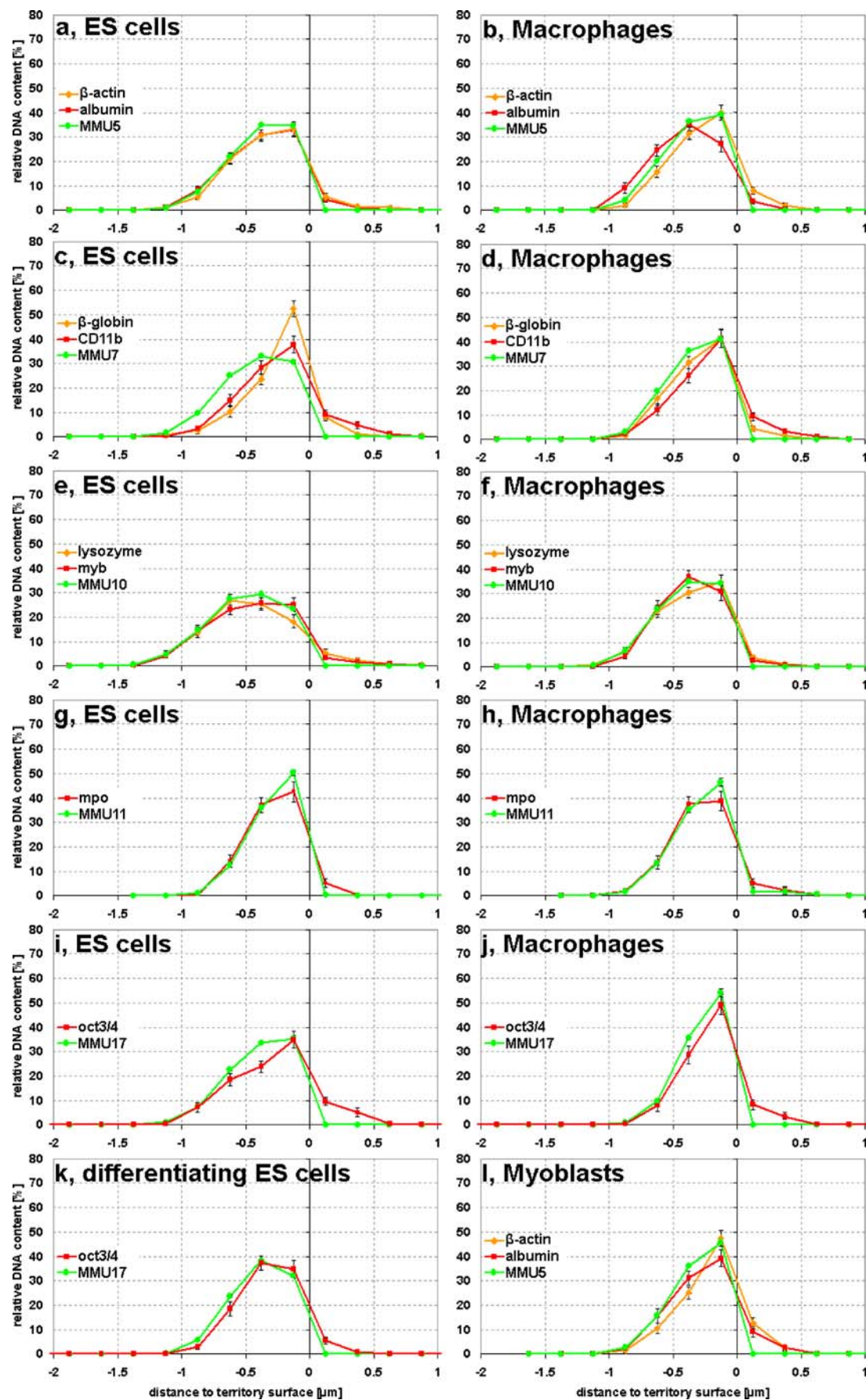
We next sought to analyze whether expressed gene loci colocalize with nuclear splicing factor speckles, as it was observed for several active genes (Nielsen et al. 2002; Shopland et al. 2003; Chuang et al. 2006; Takizawa et al. 2008). We codetected splicing speckles together with the oct3/4 gene locus in undifferentiated ES cells, in ES cells after 5 days of differentiation, and in terminally differentiated, postmitotic macrophages. While immunostaining of macrophages against the splicing factor SF2/ASF revealed a typical speckled pattern (Fig. 1f), in both kinds of ES cells, we surprisingly obtained a much finer, punctuate pattern (Fig. 1c). Control experiments on ES cells without FISH showed the same pattern (not shown). While the homogeneous punctuate pattern precluded a meaningful analysis of gene association with speckles since the closest antibody signal was always very near; the unusual nuclear

distribution of splicing factors in ES cells is an interesting observation in itself. Radial nuclear distribution of speckle signals was more internal than for DNA counterstain, the oct3/4 gene locus, and the harboring MMU17 territory in all three cell types (Fig. 2i–k).

Position of genes relative to the harboring territory

With regard to the harboring chromosome territory, several studies found a more peripheral or even external positioning for certain expressed genes or gene clusters than for unexpressed controls (Dietzel et al. 1999; Volpi et al. 2000; Mahy et al. 2002a; Williams et al. 2002). However, internal positions have also been described for expressed genes (Mahy et al. 2002b; Stadler et al. 2004) and transcription sites (Verschure et al. 1999). To investigate the positioning of gene loci relative to their chromosome territory, we compared the distance of each gene locus signal to the territory surface with the distance of territory signal voxels to the territory surface (Fig. 5).

Fig. 5 a–l Distance of FISH signals to the surface of their chromosome territory. The two territories from one nucleus were analyzed independently. The routine explained in legend to Fig. 2 for nuclear surfaces was applied here for territory surfaces. See main text for discussion



When two gene loci were detected simultaneously, they mostly showed similar distributions of distances to the territory surface (Fig. 5a–f,l; Table 5). This includes cases where we found marked differences in the radial nuclear distribution for the two loci, e.g., *myb* and *lys* (compare Fig. 2e,f with Fig. 5e,f). Thus, while the distance of these loci to the nuclear surface is significantly different due to the polarity of the chromosome territory, their distance to the territory surface is not (Table 5). These results are in agreement with a recent study on several endogenous human gene loci (Küpper et al. 2007).

Most of the gene locus signals were well within their respective territories. However, for each locus, we also found signals which were partially or completely outside the segmented territory, albeit in the immediate neighborhood less than 0.5 μm away. The fraction of the total gene locus signal which was found outside the territory amounted usually to about 10% (Fig. 5a–l). While absolute gene locus to territory surface distances are dependent on chosen territory thresholds, a relative comparison of two gene loci within the same population of chromosome territories is largely threshold independent. In ES cells, the nontranscribed β -globin locus was found more towards the periphery of the territory than the expressed CD11b locus (Fig. 5c, for expression, see Supplemental Fig. 4), although the difference was not significant. We interpret the positioning of the β -globin locus at the territory's surface as a consequence of a strong tethering to the nuclear periphery (see above).

The transcription factor *oct3/4* is expressed in ES cells but switched off permanently soon after differentiation has begun (Koestenbauer et al. 2006). In 2D FISH experiments using air-dried nuclei, the *oct3/4* gene was described in

human lymphoblastoid cells as being on average 0.03 μm inside the boundary of its chromosome territory but 0.15 μm outside in human ES cells and thus most likely outside of the territory in a majority of ES cell nuclei (Wiblin et al. 2005). A study on morphologically preserved cells found the *oct3/4* locus on extended chromatin loops, away from the harboring core territory, in 70% of undifferentiated human ES cells but at the territory periphery in differentiated human ES cells (Bártová et al. 2008). In contrast, in three-dimensionally preserved nuclei from mouse ES cells and macrophages, we found about 85% of the total signal from *oct3/4* gene loci inside the segmented boundary of the chromosome territory (Fig. 5i–k). We did observe a difference in the shape of the MMU17 territories (Fig. 5i,j, green curves): in macrophages, MMU17 has more material closer to its surface, indicating either a flatter form or more invaginations. However, in both cell types, the distribution of distances from *oct3/4* locus signals matched the one from the territory itself rather closely. The reason for the differences described for mouse and human *oct3/4* locus positioning relative to their territory may lay in a different expression of neighboring major histocompatibility complex class I region genes (see Wiblin et al. 2005 for references) or other differences between human and murine ES cells (Koestenbauer et al. 2006).

Nuclear positioning and genomic properties

At first glance, our data suggest that radial nuclear positioning may be determined not by gene density per se but by other genomic properties, usually but not always correlating with gene density, such as GC content and the presence of LTR or LINE1 elements. However, results from Bernardi and coworkers suggest that a high GC content may be an evolutionary consequence of internal localization rather than the other way round (Federico et al. 2006): In warm-blooded vertebrates, GC-rich isochores of the genome were found in central areas of the nucleus (Bernardi 2000; Saccone et al. 2002; Federico et al. 2004, 2005). Compared to cold-blooded vertebrates, warm-blooded vertebrates have an increased GC content in gene-rich regions of the genome, most likely to thermodynamically stabilize the structure of genes transcribed in many tissues by increasing the binding forces between the DNA single strands (Jabbari and Bernardi 2004). However, despite the absence of high GC content in cold-blooded vertebrates, when DNA probes of GC-rich, gene-rich sequences from chicken were hybridized to nuclei of a frog and a lizard, the homologous regions detected were found in central areas of the nuclei (Federico et al. 2006). An increase in GC content in warm-blooded animals could confer a selective advantage particularly in genes which are expressed in many

Table 5 *p* values for gene positioning relative to their territories

	Gene1–gene2	Gene1–terr	Gene2–terr
ES: <i>act/alb</i>	0.287	0.026	0.395
ES: β -glob/ <i>cd11b</i>	0.377	<0.001	<0.001
ES: <i>lys/ myb</i>	0.867	0.201	0.03
ES: <i>mpo</i>	–	0.963	–
ES: BWS	–	<0.001	–
ES: <i>oct3/4</i>	–	<0.001	–
Diff. ES <i>oct3/4</i>	–	0.008	–
Mph: <i>act/alb</i>	<0.001	<0.001	0.065
Mph: β -glob/ <i>cd11b</i>	0.087	0.025	<0.001
Mph: <i>lys/ myb</i>	0.99	0.152	0.341
Mph: <i>mpo</i>	–	0.939	–
Mph: <i>oct3/4</i>	–	0.007	–

p values were calculated for all comparisons possible within a given population of nuclei and are based on a pairwise comparison of the mean signal positions in each nucleus.

different cell types while a selective advantage may be lower or absent for genes which are functionally important only in one cell type such as the β -globin gene or the neighboring olfactory receptor genes (Bulger et al. 1999). This may be one reason why the correlation of GC content and gene density is not perfect. It should be also kept in mind, however, that all values of gene density must be considered preliminary at present. While most protein coding genes have been identified by now in mouse and human genomes; data from *Caenorhabditis elegans* suggest that there may be an equally large or even larger number of genes for other types of RNAs (Cheng et al. 2005; Ruby et al. 2006). It can only be speculated whether such RNA genes are present in similar numbers in vertebrate genomes and if so where their chromosomal location is.

Similar to GC content, LTR and LINE content may be a consequence of accumulation or better preservation of such sequences over evolutionary timescales in chromatin regions with specific properties such as “open” and “closed” chromatin rather than being a driving force in nuclear positioning. Still, GC content and certain repetitive elements may turn out to be of general value as indicators or predictors of nuclear positioning. In this context, however, it should be noted that the best correlation that we found for average radial gene positioning in macrophages was positioning in ES cells and vice versa ($R=0.83$, Fig. 4f). This high correlation for gene positioning in rather distantly related cell types suggests that additional yet unidentified chromatin features may play an important role.

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